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METHODS EVALUATION

Development of a Testing Battery to Assess Chemical-Induced Immunotoxicity: National Toxicology Program's Guidelines for Immunotoxicity Evaluation in Mice

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I. BACKGROUND

Traditional methods for toxicological assessment (i.e., histology) have implied that the immune system is a frequent target of toxic insult following subchronic or acute exposure to environmental chemicals, therapeutic drugs, abused drugs, or radiation. Interaction of these xenobiotics with the immune system may result in undesirable effects of three principal types: (1) those manifested as immunosuppression or in rare instances as enhancement; (2) those manifested as autoimmunity; and (3) those manifested as an allergic reaction in which there is a response directed against the chemical. The extent of chemical-induced hypersensitivity disease has been known for some time (Young, 1980; Luster and Dean, 1982). However, only recently has the scientific community developed an awareness that a broad spectrum of xenobiotics can suppress the immune system and decrease host resistance to infectious agents in laboratory animals (Vos, 1977; Sharma, 1981, Dean *et al.*, 1985). Several clinical studies in humans have demonstrated parallel effects on immune dysfunction. For example, it has been reported that individuals accidentally exposed to polychlorinated biphenyl-contaminated rice oil (Lee and Chang, 1985), polybrominated biphenyl-contaminated dairy products (Bekesi *et al.*, 1985), or isothiocyanate-derived imidazolidinethion-adulterated rapeseed oil (Kamuller *et al.*, 1984) exhibit immune dysfunction. These incidents are of concern because of the known association between the therapeutic use of chemical immunosuppressants and increased incidences of infectious disease (Allen, 1976), neoplastic transformation (Penn, 1985), and monoclonal gammopathies (Radl *et al.*, 1985). Whether exposure to chemical xenobiotics present in the environment affects the immunocompetence of the general population (excluding immunotherapy) has not been determined and remains a critical issue.

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The initial strategies among immunologists working in toxicology and safety assessment have been to select and apply a tiered panel of assays to identify immunosuppression or enhancement that may occur following chemical exposure. While a number of assays and panels have been described, little effort has been made to compare, refine, or validate these assays for screening purposes. The National Toxicology Program (NTP) at the National Institute of Environmental Health Sciences (NIEHS) initiated efforts to develop and validate a simple screening battery of immunological tests as an adjunct to more traditional toxicity and carcinogenicity testing. In November 1979, a consensus meeting was held at NIEHS to prioritize a list of relevant immunological assays suitable for chemical evaluation. Most of the methodologies proposed in the panel had been used successfully in diagnosis of primary and secondary immunodeficiency diseases in laboratory animals or humans. However, further validation was required to determine whether those tests selected for the screening panel were sensitive and encompassing enough to detect the more subtle immunologic alterations that could occur following chemical exposure. Furthermore, it was felt that this panel should be capable of serving as a diagnostic aid for secondary immunodeficiency disease which would allow it to be highly useful in risk assessment.

Since the most relevant endpoint for immune dysfunction is altered host resistance (e.g., to bacteria, viruses, parasites, or tumor cells) (Bradley, 1985), emphasis was placed on developing sensitive infectivity models and correlating immune aberrations with altered host resistance. When selecting assays to be utilized in the screening program, consideration was also given to miniaturization, simplicity, and reproducibility without loss of sensitivity. Development of a screening panel was accomplished primarily through an intramural research effort at the NIEHS and two methods development contracts (N01-ES-5000 and 5001) which allowed interlaboratory validation. The contract laboratories involved were the Department of Pharmacology and Toxicology, Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia, and Life Sciences Division, IIT Research Institute, Chicago, Illinois. The Cell Biology Department at the Chemical Industry Institute of Toxicology (CIIT), Research Triangle Park, North Carolina, also took part in portions of the interlaboratory validation. As part of these efforts, a number of established immunomodulators or immunotoxicants were examined. These included diethylstilbestrol (DES), cadmium, cyclophosphamide, dexamethasone, and benzo[*a*]pyrene which provided an array of effects on multiple cell types (e.g., B cells, T cells, macrophages). The following is a summary of data accumulated in these laboratories over the past 4 years, including a description of the sensitivity and reproducibility of some of the assays selected in the screening panel. This report is not meant to be a comprehensive review of these efforts, but rather an overview which should provide the reader with a general understanding of the rationale and outcome for the particular assays selected.

II. TIER APPROACH

Due primarily to the complexity of the immune system, efforts to assess chemical-induced immunotoxicity in laboratory animals have historically been performed through a tiered approach with multiple assays (Vos, 1980; Dean *et al.*, 1982a; Speirs *et al.*, 1978; Koller and Exon, 1985; Luster *et al.*, 1982). For example, immunotoxicity screening at the National Institute of Public Health and Environmental Hygiene, The Netherlands, is based primarily on assessment of immunopathology parameters included as part of a subchronic toxicity study protocol. When the results of screening studies indicate that a chemical may be immunotoxic, functional tests are performed as part of an expanded tier in the same strain of animals (Vos, 1980). In contrast, NTP's original Special Studies Panel for Immunotoxicity (Dean *et al.*,

TABLE 1

PANEL FOR DETECTING IMMUNE ALTERATIONS FOLLOWING CHEMICAL OR DRUG EXPOSURE IN RODENTS[a]

Parameter	Procedures
SCREEN (Tier I)	
Immunopathology	Hematology—Complete blood count and differential
	Weights—Body, spleen, thymus, kidney, liver
	Cellularity—Spleen
	Histology—Spleen, thymus, lymph node
Humoral-mediated immunity	Enumerate IgM antibody plaque-forming cells to T-dependent antigen (SRBC). LPS mitogen response
Cell-mediated immunity	Lymphocyte blastogenesis to mitogens (Con A) and mixed leukocyte response against allogeneic leukocytes (MLR)
Nonspecific immunity	Natural killer (NK) cell activity
COMPREHENSIVE (Tier II)	
Immunopathology	Quantitation of splenic B and T cell numbers
Humoral-mediated immunity	Enumeration of IgG antibody response to SRBCs
Cell-mediated immunity	Cytotoxic T lymphocyte (CTL) cytolysis. Delayed hypersensitivity response (DHR)
Nonspecific immunity	Macrophage function-quantitation of resident peritoneal cells, and phagocytic ability (basal and activated by MAF)
Host resistance challenge models (endpoints)[b]	Syngeneic tumor cells
	PYB6 sarcoma (tumor incidence)
	B16F10 melanoma (lung burden)
	Bacterial models
	Listeria monocytogenes (mortality)
	Streptococcus species (mortality)
	Viral models
	Influenza (mortality)
	Parasite models
	Plasmodium yoelii (Parasitemia)

[a] The testing panel was developed using B6C3F1 female mice.
[b] For any particular chemical tested only two or three host resistance models are selected for examination.

1982a) included a limited number of functional and host resistance assays as part of the initial screen. The present NTP configuration, which will be discussed in detail later, is composed of two tiers (Table 1). Tier I is a limited effort and includes assays for both cell-mediated immunity (CMI) and humoral-mediated immunity (HMI), as well as immunopathology, the latter of which is part of the standard protocol in NTP carcinogenicity/toxicity subchronic studies.

While the probability of detecting potent immunotoxicants in Tier I is high, the likelihood of detecting weaker immunotoxicants, such as those that may effect only a specific cell population or subpopulation, is presumably less. Nonetheless, based upon the data from compounds which have completed both Tier I and II testing, no compound has been found to effect an assay in Tier II without demonstrating some effect in Tier I. Thus, while Tier I provides little information on the specificity of immune defect or its relevance to the host, it can readily discern an immune alteration resulting from chemical exposure. Histopathological and gross changes in lymphoid organs evaluated in Tier I are heavily relied upon as supportive evidence for further investigation, and detection may depend upon the skill with which the pathologist can identify changes of potential immunotoxic significance. The antibody plaque-forming cell (PFC) response to sheep red blood cells (SRBCs) appears to be the most

commonly effected functional parameter in animals exposed to chemical immunosuppressants. The immunological phenomena associated with antibody production and the accumulated laboratory data demonstrating the reproducibility and sensitivity of this response are discussed later. Several mitogenesis assays are also included in the Tier I testing panel. The mixed leukocyte response (MLR), which measures proliferation, appears fairly sensitive in detecting immune effects, probably because it reflects an antigen-driven system. Although mitogenesis assays with polyclonal activators such as the plant lectin concanavalin A (Con A) or bacterial products such as lipopolysaccharide (LPS) are generally not very sensitive, they have been included in the Tier I panel since they correlate with specific changes in host resistance (see Table 7) and can easily be performed in tandem with the MLR assay with minimum additional effort. At present Tier I does not include a T-effector-cell function test, since an effector test that is reproducible, simple, and sensitive enough to meet Tier I requirements has not been found. However, MLRs were found to closely correlate with T-cell-dependent host resistance models (see Table 7) and provide a good indication of T-cell function. Delayed hypersensitivity responses (DHRs), although commonly used in humans, do not appear to be highly sensitive in the mouse, while quantitation of allograft rejection time is too time consuming for a screening panel. The cytotoxic T-lymphocyte response (CTL) appears to meet the criteria necessary for inclusion into Tier I, but has not undergone an interlaboratory validation effort. At the present time the DHR and CTL assays are included in Tier II and are performed when T-cell defects are noted in Tier I. Also included in Tier I is the quantitation of natural killer (NK) cell function in the spleen. This population is examined since NK cell function does not overlap with HMI or CMI and certain xenobiotics have been shown to selectively effect NK cells (Luster *et al.*, 1987; Rogers *et al.*, 1983).

Tier II, which represents an in-depth evaluation, includes additional assays for CMI, HMI, and nonspecific immunity, as well as an examination of host resistance. Tier II testing is normally included only if functional changes are seen in Tier I and at dose levels which are not overtly toxic (i.e., body weight changes). Immune function tests in Tier II will provide information on the mechanism of the immunotoxicity and help characterize the nature of the effect (e.g., determining the subpopulation affected). Quantitation of B- and T-cell numbers, including T-cell subpopulations (i.e., Lyt 2 and L3T4), will aid in determining whether a chemical acts via a specific functional modulation or a cellular depletion mechanism. Evaluation of IgG antibody responses will aid in determining whether secondary responses are affected, while assays for CMI can detect whether effector cell function is affected. Measures for nonspecific immunity in Tier II focus on macrophage function and at present include the quantitation and phagocytic ability of peritoneal cells. The latter test is performed *in vitro* in the presence and absence of macrophage activating factor (MAF). Macrophage function tests have been the most difficult to incorporate into a testing scheme for several reasons, including inability to isolate sufficient cell numbers, sources, assay variability, and relationship to relevant endpoints. While further development of macrophage screening tests is required for any test selected it will be important to determine whether the chemical modulates the activity of resting macrophages as well as alters their ability to become activated in response to stimuli, an approach proposed by Adams *et al.* (1983). Also included in Tier II are a series of assays in which the ability of animals to resist challenge with infectious agents or transplantable tumor cells is measured. Since it is not realistic or necessary to perform all the host resistance assays listed in Tier II, only selected infectivity models (i.e., two or three agents at most) are examined. Selection of appropriate challenge models is based upon both experimental considerations and results obtained under

functional examination (Tier I). For example, the B16F10 tumor lung burden or influenza challenge assays would be selected for those compounds administered via inhalation since resistance to these agents involves, in part, local immune defense mechanisms in the lung. For those compounds shown to affect T-cell functions in Tier I, challenge assays such as *Listeria* or PYB6 may be appropriate since resistance to these agents involve competent CMI.

Because considerable effort is required for immunological assessment, evaluation is not included as part of the standard carcinogenicity/toxicity testing. Instead, chemicals to be examined for immunotoxicity are selected following consideration of such factors as structure–activity relationship, pharmacokinetic properties, changes in lymphoid organ weights or histology, hematologic parameters, and, if available, tumor types and incidences obtained from previous *in vivo* studies of the chemical. A positive control group is normally included in the tier, which is composed of age- and sex-matched mice administered a single ip injection of 200 mg/kg of freshly prepared cyclophosphamide 48 hr before testing. This concentration of cyclophosphamide normally results in significant inhibition of most of the functional tests, and provides an indicator of assay reproducibility and of relative "immunotoxic potency" for the chemical being tested.

Since the panel represents a screen, certain limitations exist. Animals are normally evaluated at only one specific time point, thus there is no routine measure for chemical tolerance or reversibility of immunological changes. Furthermore, there are no measurements of specific immunological compartments, particularly lung immunity, which may be the primary target affected following inhalation exposure (Bice, 1985). However, the panel does provide sufficient observations to determine directions for further study. Furthermore, assays in the panel have sufficient overlap so that immunotoxicity need not be defined as an alteration in a single parameter, but rather as a profile similar to, but certainly not to the same degree as, that used to establish AIDS (Hersh *et al.*, 1986). While developed to assess immunosuppression, the assay panel is readily adaptable for identifying compounds which may enhance immune function. With respect to the latter, challenge is performed using an LD80 or TD80 of infectious agent or tumor cells, respectively, rather than an LD20 or TD20.

III. PROTOCOL DESIGN

While the immunology data obtained from the screening panel represent an independent evaluation of a specific organ system, the experimental data are designed as an adjunct to prechronic and chronic toxicity and/or carcinogenicity testing performed by the NTP. Thus, the experimental design (e.g., dose, route, frequency) is dictated by the prechronic or chronic toxicity study protocols. This, in turn, is based upon such factors as route of human exposure, pharmacokinetic properties of the chemical, and attempts to establish no-observable-effect levels. We routinely employ a 14-day repeat-dose regimen to assess chemical-induced immune alterations. However, acute, 30- and 90-day exposure regimens have been utilized at various times when dictated by the pharmacokinetic properties of the chemical being evaluated. As previously indicated, immunotoxicity testing is not included as part of the standard NTP protocol for carcinogenicity/toxicity testing, but rather is employed on a selective basis.

B6C3F1 mice are used for immunological evaluation since this species and strain is frequently used by the NTP for toxicity and carcinogenicity testing, not because of any specific immunological considerations. In fact, most of the immune assays used are adaptable to inbred strains of rats (Koller and Exon, 1985). Utilization of inbred strains of rodents is recommended since this will decrease the variation between individual animals compared to randomly bred individuals. Since it was decided to validate the meth-

ods in only one species, there were several advantages of using the mouse over other species, including the rat. First, more historical control data were available in the mouse than other species. Second, immunological reagents for the mouse were readily available to monitor biological markers for identification of cell populations, cell function, and maturational status. Third, there was a large background of genetic data in the mouse not readily available in other species which provided information regarding ability to develop immune response to specific antigens (e.g., *Ir* genes) and resistance to infectious agents. Finally, syngeneic tumors and models of susceptibility to specific infectious agents were more readily available in the mouse than they were in other species.

IV. METHODOLOGY OVERVIEW

(1) Immunopathology

Procedures used to detect immune alterations which can be incorporated into a routine toxicology design include histology of thymus, lymph node, and spleen; spleen and thymus weights; complete blood count (CBC) and differential; and splenic cellularity. Routine histopathology of lymphoid organs may be useful in assessing the immunomodulatory effect of a chemical, particularly when these data are combined with changes in weight and cellularity of the thymus and/or spleen. The structural division of the spleen and lymph nodes into thymus-dependent and thymus-independent areas necessitates careful microscopic examination and the use of specific staining techniques may indicate preferential effects of the chemical on T- or B-cell numbers (Irons, 1985).

The expression of specific receptors and antigenic determinants on the cell surface of lymphocytes and macrophages has been used in the diagnosis of immunodeficiency diseases. Specific antibody reagents can be used for qualitative (e.g., functional status) and quantitative analysis of B cells, T cells, stem cells, and macrophages. As part of Tier II, splenic B cells and T cells are enumerated by microscopic or cytometric analysis using fluorescent-labeled antibodies to cell surface antigens including sIg and Thy 1.2 and when appropriate Lyt 2 and L3T4.

(2) Humoral-Mediated Immunity

(a) Antibody response. The PFC assay quantitates the production of specific antibody through enumeration of antibody-producing cells following immunization with an antigen (Cunningham, 1965). The most common antigen employed experimentally is SRBCs. The response to SRBCs requires the cooperation of a number of cell populations, including B cells, T helper cells, and macrophages. Macrophages are required for antigen processing as well as for production of interleukin 1. T cells aid in antigen recognition via surface membrane proteins, as well as in B-cell maturation via the production of mediators required for cell proliferation (e.g., B-cell growth and stimulatory factors) and differentiation (e.g., B-cell differentiation factors). T cells are also involved in regulation of the isotype switch. The kinetics for the development of the optimal IgM and IgG responses, concentration of SRBCs, route of injection, and ranges have been determined for female B6C3F1 mice (see Table 5 and Thomas *et al.*, 1985a,b). Spleen cell numbers are routinely obtained and results are reported as PFC/spleen as well as PFC/10^6 nucleated viable cells in order to correct for chemical-induced changes in splenic cellularity and to convey functional ability of the cell vs a "whole animal" effect.

(b) Lymphoproliferative responses to LPS. Lymphocyte activation and proliferation represent sequential steps in the development of humoral immune responses reflecting transition from G_0 to G_1 and S phase of the cell cycle. B lymphocytes can be polyclonally stimulated to proliferate in the presence of a variety of bacterial cell products, including LPS and this is quantitated by the incorporation of [^{3}H]thymidine into DNA (Anderson *et al.*, 1972). Suppression of the LPS mitogen response does not necessarily indicate that functional impairment of B cells has occurred and in fact LPS and PFC responses may not always correlate. This is likely due to the fact that LPS mitogenesis represents only a "window" in B-cell maturation and, unlike antigen-induced maturation, does not measure early events in activation or in terminal differentiation of B cells into antibody-secreting cells (Klaus and Hawrylowicz, 1984). It is becoming more apparent that proliferation (mitogenesis) assays using polyclonal activators, such as LPS or Con A, are not highly sensitive when compared to antigen-driven systems. We have elected, however, to leave these assays in the panel since mitogenesis can be performed in tandem with the MLR with little additional effort and provides information on the proliferative capacity of the respective subpopulation.

(3) Cell-Mediated Immunity

(a) Lymphoproliferative responses to Con A. In acquiring a cell-mediated immune response, sensitized T cells undergo blastogenesis and proliferation in response to specific antigens. T cells also undergo blastogenesis and proliferation in response to polyclonal activators such as the plant lectins Con A or phytohemagglutinin (PHA). As described for LPS-induced proliferation, these

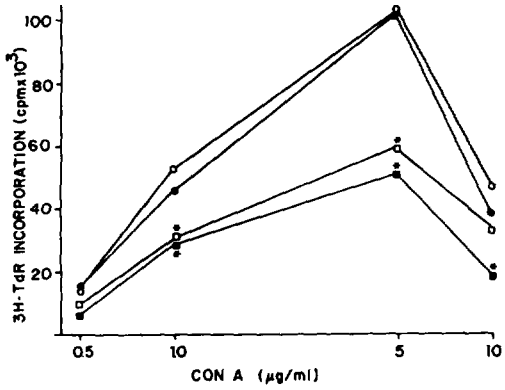

FIG. 1. Effects of increasing Con A concentration on the lymphoproliferative response in control and nitrobenzene-treated mice. Female B6C3F1 mice were administered corn oil (O) or nitrobenzene by gavage daily for 14 days at dose levels of 30 (●), 100 (□), or 300 (■) mg/kg body wt. Values represent mean cpm from at least seven animals per treatment group. Significant at $p < 0.01$ versus controls.

responses are measured by the uptake of [^{3}H]thymidine into DNA. The use of both Con A and PHA may give some indication as to the maturational status of the T lymphocyte pool sensitive to perturbation, since Con A-responsive cells are relatively immature as compared to PHA-responsive T cells (Stobo and Paul, 1973). However, in our experience, similar responses have generally been obtained and usually only Con A is tested. As with LPS, the effect of xenobiotics on T-cell lymphoproliferation is determined using a range of mitogen concentrations which ensures that an optimal response is obtained (Luster *et al.*, 1982). As shown in Fig. 1, the ability to detect quantitative changes in mitogenesis following exposure to nitrobenzene is directly proportional to the mitogen concentration employed. Chemical-induced suppression was most evident at the optimal concentration, which in this case was 5.0 Mg/ml of culture media for Con A. This has been found to be the case with most chemicals tested.

(b) Mixed leukocyte response (MLR). The lymphoproliferative response of spleen cells to allogeneic spleen cells has provided a very sensitive indicator for CMI to detect chemical-induced immunosuppression. From a clinical viewpoint, the MLR measures the same response involved in graft vs host reactions and graft rejection, and has been shown to be predictive of host response to transplantation and of general immunocompetence (Harmon *et al.*, 1982). Since both populations of spleen cells in the MLR are capable of recognition and subsequent response, a unidirectional MLR is preferred whereby the stimulator cells are inactivated by mitomycin C or radiation treatment prior to addition to culture (Bach and Voynow, 1966). As with mitogen-induced blastogenesis, stimulation by allogeneic leukocytes is quantitated by [^{3}H]thymidine uptake. However, the period of incubation is increased from 3 to 4 days and culture conditions have been slightly modified to obtain optimal responses in B6C3F1 mice (Luster *et al.*, 1984).

(c) Generation of CTL. A direct follow-up to the MLR evaluation of effector cell function is the cytotoxic T-cell assay. Interaction of T cells with tumor or MHC antigens results in the acquisition of IL-2 reactivity consequent to the expression of IL-2 receptors. In the presence of IL-2, these cells develop into fully cytotoxic T cells (i.e., capable of lysing syngeneic or allogeneic tumor cells). The ability of splenocytes to generate CTL is assessed by a modification (Murray *et al.*, 1985b) of a previously described method using P815 tumor target cells (Brunner *et al.*, 1976).

(d) DHR to keyhole limpet hemocyanin (KLH). The DHR represents an *in vivo* assessment of the sequential steps necessary for a cell-mediated immune response. This includes antigen recognition and processing, blastogenesis and proliferation, migration of memory T cells to the site of inflammation (challenge), and production and release of inflammatory mediators and lymphokines, culminating in an inflammatory response. Radioisotopic procedures have been developed to measure either vascular permeability or monocyte influx (Lefford, 1974). We have modified the latter so that it may be successfully used in B6C3F1 mice using an [^{125}I]UdR technique (Holsapple *et al.*, 1984). The magnitude of the DHR can be reported as a stimulation index (S.I.), which is calculated for each sensitized animal as a ratio of the monocyte influx at the challenge site (ear) to the monocyte influx into a comparable unchallenged site. To control for large differences in control ear responses, the DHR can also be reported as the difference between the control and sensitized ears corrected for sensitization controls. In our experience the DHR is not as sensitive as the MLR or CTL responses for detecting immune suppression. This is exemplified in Table 2 where the effects of exposure to subcutaneous injections of phorbol myristate acetate (PMA) were examined on various CMI functions. Mice were given subcutaneous injections of PMA for 2 weeks at total exposure levels of 2, 20, or 40 mg/kg. PMA, a weak inhibitor of CMI, suppressed the MLR, Con A, and CTL responses without effecting the DHR. Nonetheless, provided that a certain degree of immunodeficiency occurs, the DHR test is a good overall assessment for CMI which correlates with immunodeficiency and decreased resistance to infectious disease in both experimental animals and humans (MacLean, 1979).

(4) Nonspecific Immunity

(a) Natural killer (NK) cell activity. NK cells possess innate cytotoxicity against a variety of neoplasms, inhibiting both growth and metastatic dissemination of tumor cells in addition to various infectious agents (reviewed by

TABLE 2

EFFECT OF PMA ON CELL-MEDIATED IMMUNE FUNCTIONS[a]

Dose (mg/kg)	CTL function[b] Percentage cytotoxicity (% decrease)	DHR index[c]	MLR[d]	Con A[d]
0	86[d]	2.9 ± 0.2	14 ± 1	66 ± 3
2	84 (2)	3.3 ± 0.3	15 ± 2	69 ± 6
20	74 (14)	3.4 ± 0.4	15 ± 1	53 ± 7
40	63 (27)*	3.2 ± 0.4	10 ± 1*	33 ± 3*

[a] Mice were administered four subcutaneous injections of PMA over 2 weeks for a total of 2, 20, or 40 mg/kg. Each value represents the mean ± SE of at least six mice per group.

[b] Mean percentage cytotoxicity of P815 tumor target at 25:1 effector target cell ratio.

[c] Indices were calculated from DPM (sensitized ear) − background/DPM (control ear) − background.

[d] Values represent CPM × SE (×10³) [³H]TdR incorporation in splenic lymphocytes stimulated with either MMC-treated spleen cells from DBA mice in a MLR or Con A.

* $p < 0.05$ vs controls by ANOVA and Dunnett's multiple range test.

Herberman, 1985). NK cell activity is quantitated from spleens of individual mice using an *in vitro* ^{51}Cr release assay in which YAC-1 tumor cells are used as the target cell (Reynolds and Herberman, 1981). Because this assay can be miniaturized in the microtiter plate several effector:target ratios are routinely assayed (e.g., 50:1 and 100:1).

(b) Macrophages. Determination of resident peritoneal cell number and differential count is a rapid and simple, although not very sensitive, means of assessing the effects of nonparentally administered xenobiotics on macrophage function. Several immunotoxic chemicals such as DES and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), as well as clinical immunodulators, change the differential of resident peritoneal cell counts (Lewis and Adams, 1985). Immunotoxicants can also shift the differential count of peritoneal exudate cells (PECs) which are recruited through the use of eliciting agents such as *Corynebacterium parvum*, MVE-2, or thioglycollate (Dean *et al.*, 1984). The phagocytosis of foreign material, including pathogens, by macrophages and polymorphonuclear cells (PMNs) represents a major first-line defense mechanism in nonspecific immunity. A rapid means of measuring phagocytosis has been to utilize fluorescent latex beads which, when added to cultures containing adherent PECs, are ingested by phagocytic cells. By observing this preparation under a fluorescent microscope or by flow cytometry, the percentage of cells which have phagocytized beads can be readily determined (Duke *et al.*, 1985). When monitoring the effects of chemicals on macrophage function, it is important to determine whether the chemical modulates the ability of the macrophage to become activated (e.g., *in vitro* via MAF activation or *in vivo* following treatment with *C. parvum*). Several macrophage assays were evaluated that provided information on more defined endpoints regarding function and activation state than phagocytosis such as antitumor activity, antibacterial activity, and ectoenzyme levels (Morahan *et al.*, 1980; Thomas *et al.*, 1985b). However, these assays were labor intensive and somewhat variable, thus, not lending themselves to screening efforts.

(5) Host-Resistance Assays

Altered susceptibility to infection or tumor cell challenge can be an *in vivo* manifestation of chemical-induced immunotoxicity. To document impaired host defenses, a concentration of infectious agent or tumor cells which will produce an LD10–30 or TD10–30, respectively, in control mice is administered. To determine if the chemically treated animals are immune to reinfection, a much larger secondary challenge dose (equivalent to an LD60–80 for primary challenge) can be administered to survivors. Alternatively, an LD60–80 or TD60–80 can be used to examine potential immunoenhancers. The host-resistance models discussed below were chosen from a large group of assays initially evaluated for the following reasons: (1) the mechanisms of resistance are well understood and generally do not overlap; (2) the models have proven to be reproducible and are technically easy to perform (Morahan *et al.*, 1984; Dean *et al.*, 1982b; Fugmann *et al.*, 1983), and (3) they represent endpoints which are relevant to human health.

(a) Listeria monocytogenes. The pathogenesis and immune mechanisms involved in *L. monocytogenes* infection have been studied extensively. Recovery from infection depends upon specific sensitization of T cells which subsequently activate macrophages for enhanced nonspecific bactericidal activity (North, 1973). Initially, fixed macrophages are involved in inactivation of the bacterium, while the T-cell-mediated activation of macrophages occurs 2–3 days following systemic infection. Resistance to the bacterium can be assessed by mortality or by quantitation of bacteria in the liver and spleen, which are the major sites for replication. While resistance of mice to *L. monocytogenes* is genetically determined, the mouse strain used appears to influence only the number of organisms required to induce lethality or disease, not the final outcome. The C57BL/6 background of the B6C3F1 mouse confers resistance, which necessitates that a greater number of organisms are required for the LD20 than if a more susceptible strain is used.

The assay is performed using previously established techniques (Vos, 1980; Fugmann *et al.*, 1983; Pung *et al.*, 1984; Holsapple *et al.*, 1985). The bacteria are normally administered by iv inoculation where growth can be detected in the liver and spleen within 24–72 hr. Mortality from a lethal inoculum of *L. monocytogenes* occurs be-

tween 5 and 14 days. *Listeria* is not a normal pathogen in the mouse and is not transmitted horizontally. However, it is pathogenic in human neonates and can be considered an opportunistic bacterium since it may produce disease in immunocompromised individuals.

(b) Streptococcus species. The immune mechanisms associated with resistance to *Streptococcus* infection are dependent upon the induction of opsonizing antibody, which results in enhanced phagocytosis of *Streptococcus spp.* and subsequent intracellular destruction (Bradley and Morahan, 1982). PMNs and serum complement also play a role, particularly during the early phase of the infection (Winkelstein *et al.*, 1981). Thus, this host-resistance model reflects the ability to produce a T-independent antibody response (i.e., to the streptococcal polysaccharide), the functional capacity of granulocytic and phagocytic cells, and serum complement activity.

The assay has been performed using several streptococcal strains including *S. pneumoniae* (White *et al.*, 1986), a smooth encapsulated strain that is pathogenic for mice, and *S. zooepidemicus* (Fugmann *et al.*, 1983). The successful use of *Streptococcus* (as well as *Listeria*) is dependent upon obtaining the organism when in exponential growth as well as an accurate estimation of the number of viable organisms inoculated, which in turn can be determined by spectrophotometry and/or plate counts. The number of organisms required to produce lethality and the time to death following inoculation is dependent upon the species. In the case of *S. pneumoniae* in mice, the dose is large (2×10^7) and the time to death for control mice is short indicating a possible role for PMNs and complement. In contrast, the time to death following injection with *S. zooepidemicas* is considerably longer which probably reflects antibody-mediated resistance. *S. pneumoniae* is pathogenic for man and should be handled with caution. Pathogenicity in mice is highly dependent on the strain, but horizontal transmission has not been observed between B6C3F1 mice in our experience.

(c) PYB6 tumor. The PYB6 fibrosarcoma tumor was originally induced with polyoma virus in C57BL/6 mice and was developed as a screening model in the B6C3F1 mouse (Dean *et al.*, 1982b). Resistance to tumor growth, which reflects CMI and NK cell function (Urban *et al.*, 1982; Murray *et al.*, 1985a), is measured by differences in tumor frequency following subcutaneous challenge into the thigh with $1-5 \times 10^3$ viable tumor cells (approximate TD10–30). Tumor cells for challenge should be obtained from an animal bearing a 10- to 12-mm tumor rather than from tissue culture. However, the PYB6 cell line can be maintained for fairly long periods in culture or frozen, but should be passed in an animal prior to challenge studies. Due to antigenic shifts in the tumor cells, adjustment of challenge cell inoculum is required periodically to ensure that a TD10–30 is obtained. Mice are palpated weekly and the incidence, latency (time to tumor appearance), and tumor size are recorded.

(d) Metastatic pulmonary tumor B16F10. The B16F10 tumor was selected because it is syngeneic in the C57BL/6 mouse, has weak tumor-specific antigens, and is well characterized with regard to metastasis and the response to immunotherapeutic and chemotherapeutic agents (Fidler *et al.*, 1978). NK cells and macrophages are probably the major immune mechanisms responsible for clearance and growth inhibition of pulmonary tumors, but T cells also play a role (Parhar and Lala, 1987). To perform this assay, approximately 1×10^5 B16F10 tumor cells derived from tissue culture are injected iv into control and chemically treated mice. Twenty-one days later the mice are injected ip with 50 mg/kg of fluorodeoxyuridine to block *de novo* synthesis of nucleotides, followed 1 hr later with 1 MCi of [^{125}I]UdR. Following a 24-hr labeling period the lungs are removed, pulmonary tumors are enumerated visually, and the tumor burden is determined by quantitating the incorporation of [^{125}I]UdR into the lungs as described previously (Murray *et al.*, 1985a). The use of [^{125}I]UdR incorporation to measure tumor burden is more sensitive and less time consuming than enumerating tumor nodules. As shown in Table 3, good reproducibility was observed between laboratories for the several chemicals tested. The increased resistance in lung tumor formation that occurred in DMN-exposed mice (Table 3) was due to increased uptake of tumor cells by Kupffer's cells precipitated by a chemical-induced inflammatory response. As with all the host-resistance models, this assay can quantitate enhanced resistance by increasing the challenge dose.

(e) Mouse malaria. Plasmodium yoelii, which produces a self-limiting parasitemia in mice, represents a complex host-resistance model that has potential for demonstrating both immune and nonimmune events (Krier and Green, 1980). Impairment of specific antibody production and, to some extent, T-cell and macrophage functions can contribute to decreased resistance to the parasite. Increased numbers of reticulocytes, as occurs following exposure to myelotoxic compounds, can also affect resistance by providing additional host cells for protozoan growth. This assay is performed using a nonlethal strain of *P. yoelii* (17 XNL). Mice are inoculated with 10^6 parasitized erthyrocytes within several days following chemical exposure. Blood is taken for determining of the number of parasites on Days 10, 12, and 14 after inoculation, with peak infection usually occurring on Day 12 in control mice (Luster *et al.*, 1985). Blood samples are normally analyzed by enumeration of blood smears (Dockrell and Playfair, 1983) but determination of cell size or enumeration of acridine orange-stained cells by flow cytometry has been used to discern infected from noninfected cells (Whaun *et al.*, 1983). This organism is not infectious for man and can be transferred within the mouse colony only by exposure to contaminated blood.

(f) Influenza virus. Influenza virus A2/Taiwan H_2N_2 has been used as a viral challenge model in mice to evaluate non-T-cell-mediated host defense mechanisms since both antibody (Vireligier, 1975) and interferon (Hoshino

EFFECT OF

Chemical treatment do (mg/kg)
Experiment 1[a]
Control
Cyclophosphamide (1
DES (8)
PMA (2)
Experiment 2[a]
Control
DMN (1.5)
DMN (3.0)
DMN (5.0)

[a] In Experiment 1 eac
in Experiment 2 to test
[b] Each value represen
the last exposure (Day
and were euthanized 1
[c] ND, not done.
* $p < 0.05$ vs control

et al., 1983) play major
sistance to infection in
(Fenters *et al.*, 1979),
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TABLE 3

EFFECT OF CHEMICAL TREATMENT ON GROWTH OF B16F10 TUMOR CELLS IN THE LUNG

Chemical treatment dose (mg/kg)	Lab A		Lab B	
	Nodules/lung	cpm/lung	Nodules/lung	cpm/lung
Experiment 1[a]				
Control	0[b]	239 ± 15	3 ± 1	623 ± 269
Cyclophosphamide (180)	750*	6359 ± 873*	18 ± 4*	2863 ± 108
DES (8)	0	269 ± 32	1 ± 0	219 ± 119
PMA (2)	750*	1808 ± 429*	ND	ND
Experiment 2[a]				
Control	100 ± 15	ND[c]	112 ± 34	ND
DMN (1.5)	9 ± 3*	ND	49 ± 27*	ND
DMN (3.0)	7 ± 2*	ND	24 ± 18*	ND
DMN (5.0)	5 ± 3*	ND	8 ± 7*	ND

[a] In Experiment 1 each mouse received 5×10^4 B16F10 tumor cells while higher concentrations were administered in Experiment 2 to test for increased resistance.

[b] Each value represents a minimum of six mice per group intravenously injected with tumor cells 2–5 days following the last exposure (Day 0). Mice received 50 mg/kg FUdR on Day 20 followed at 1 hr by 1×10^6 cpm of [^{125}I]UdR and were euthanized 18–20 hr later.

[c] ND, not done.

* $p < 0.05$ vs controls.

et al., 1983) play major roles in mediating resistance. Resistance to infection in the mouse is assessed by mortality (Fenters *et al.*, 1979), which occurs within 14 days following challenge in B6C3F1 mice (Thomas *et al.*, 1985b). Since this infection involves immune mechanisms in the lung, it may be advantageous to use influenza over streptococal challenge following chemical exposure by inhalation. Mortality and time to death are monitored daily for 14 days. This organism is infectious for man, but can be handled safely with moderate precautions. Proper animal husbandry procedures need to be followed to ensure that the virus is not inadvertently transmitted within the mouse colony.

V. HISTORICAL RANGES AND INTERLABORATORY VALIDATION

The major efforts in developing this screening panel were the selection of the appropriate tests to assess immunological functions as well as the development and validation of host resistance models. For immune function tests, clinical experience has established that the standardization of reagents and methodology greatly aid in the diagnosis of immunodeficiency diseases (Batty and Torrigiani, 1976). For each of the immunological assays evaluated, a variety of factors were considered which are consistent with current testing procedures (Palmer and Cavallaro, 1976). These include (1) intra- and interlaboratory reproducibility; (2) accuracy, as defined by the ability to obtain known or theoretical optimal responses from historical data; (3) assay sensitivity as determined, in part, by obtaining dose–response curves; and (4) predictivity, as judged by correlations with other functional and host resistance tests. Successful fulfillment of these criteria is essential in chemical-risk assessment for establishing an accurate database of chemical "immunotoxicants" as well as for providing a grading system for comparison and human-risk assessment. To establish these criteria for the various assays selected in the developmental phase of this project, known immunosuppressive compounds were evaluated in at least two of the participating laboratories, and in most instances three. The compounds selected for methods development and validation efforts were, among others, cyclophos-

TABLE 4

APPROXIMATE RANGE OF CONTROL VALUES FOR EACH ASSAY BY LABORATORY

Parameter	Lab A	Lab B	Lab C
Humoral immunocompetence			
Anti-SRBC IgM Day 4			
per 10^6 spleen cells	1340–3620	1200–3500	900–2450
per spleen $\times 10^3$	220–400	250–500	190–310
Anti-SRBC IgG Day 5			
per 10^6 spleen cells	1564–3686	2205–4705	ND[a]
per spleen $\times 10^3$	375–550	400–600	ND
Lymphoproliferation			
LPS (cpm $\times 10^3$)	19–59	18–60	16–91
Cell-mediated immunocompetence			
Delayed hypersensitivity response to KLH			
Stimulation index	1.7–4.9	2.0–6.0	ND
Lymphoproliferation			
Con A (cpm $\times 10^3$)	22–104	40–180	43–176
Mixed lymphocyte response (Day 5) (cpm $\times 10^3$)			
Responder only	0.4–2.1	2.2–4.9	0.3–2.1
Responder + stimulator	11–31	20–45	26–41
Nonspecific immunity			
Natural killer cell activity (percentage cytotoxicity at E:T ratio of 100:1)	6–25	3–20	7–28

[a] ND, not done.

phamide, DES, benzo[*a*]pyrene, dimethylnitrosamine, and cadmium chloride. Since these chemicals have documented general and specific immunological effects, they provided the opportunity to validate each of the immune function and host-resistance assays. All studies were conducted in B6C3F1 female mice under similar conditions and experimental protocols in the testing laboratories. Table 4 shows the range of control values for various immune function tests obtained during these studies in the participating laboratories. Not obvious from the table is the fact that the reproducibility and accuracy between the laboratories increased as the laboratories developed expertise in these procedures. The major source for the wide range in control values initially observed was due to differences in the reagents employed (e.g., source and lot of serum, source and purity of mitogens). For example, a large degree of variability was initially seen between the laboratories in the antibody PFC responses obtained in control mice (Table 4). The control response variability decreased, however, as the laboratories standardized both procedures and reagents (e.g., standardized protocols, use of high-titer lots of SRBCs, and specific complement sources). Differences in cell preparation procedures or individuals performing the assays had only minimal effects on the results.

Table 5 summarizes the results of the splenic IgM PFC response from three laboratories that evaluated five test xenobiotics over a 3-year period. Two of the xenobiotics (cadmium chloride and benzo[*a*]pyrene) were evaluated in two laboratories. The first study of DES by Laboratory A showed an unusually high control response. In the case of cyclophosphamide, the control response in Laboratory C was low (890 PFC/10^6). In both cases these chemicals were studied prior to optimization. For the most part, control values have ranged between 1000 and 2000 PFC/10^6 spleen cells during the past several

TABLE 5

INTERLABORATORY VALIDATION OF SPLENIC IgM ANTIBODY-FORMING CELL ASSAY

Treatment	Dose (mg/kg)	SRBC IgM PFCs/10^6 spleen cells ± SE (percentage change)[a]		
		Lab A	Lab B	Lab C
Diethylstilbestrol	Control	3872 ± 498	2025 ± 102	1674 ± 107
	0.1	3620 ± 524 (−9)	2380 ± 180 (+18)	ND
	1.0	3335 ± 573 (−14)	1790 ± 110 (−12)	1648 ± 80 (−2)
	4.0	1867 ± 361* (−52)	1402 ± 60* (−31)	738 ± 135* (−55)
Benzo[a]pyrene	Control	1981 ± 270	1510 ± 146	ND
	5	2038 ± 549 (+2)	1133 ± 155 (−25	ND
	20	1039 ± 231* (−48)	984 ± 191 (−35)	ND
	40	636 ± 140* (−68)	378 ± 60 (−75)	ND
Dimethylnitrosamine	Control	1898 ± 135	ND	1063 ± 103
	1.5	1424 ± 275 (−25)	ND	797 ± 150 (−25)
	3.0	728 ± 322* (−61)	ND	514 ± 71* (−52)
	5.0	599 ± 249* (−69)	ND	114 ± 94* (−91)
Cyclophosphamide	Control	1685 ± 95	1982 ± 190	890 ± 130
	45	ND[b]	ND	750 ± 130 (−7)
	90	425 ± 33* (−75)	510 ± 42* (−74)	230 ± 66* (−75)
	180	2 ± 2* (−99)	7 ± 3* (−99)	10 ± 1* (−99)
Cadmium chloride	0	1801 ± 299	ND	2226 ± 373
	12	2245 ± 495 (+24)	ND	1592 ± 195 (−28)
	20	2243 ± 362 (+25)	ND	1721 ± 122 (−23)
	30	2391 ± 324 (+33)	ND	1894 ± 150 (−15)

[a] Within 3 days following completion of the exposure, mice were immunized iv with 0.2 ml of PBS containing 5 × 10^8 SRBCs. Mice were sacrificed 4 days later and the number of IgM PFCs was determined.

[b] ND, not done.

* $p < 0.05$ vs controls.

years in all participating laboratories. Most importantly, both the percentage and magnitude of change resulting from chemical exposure were similar at each dose level between laboratories. Overall, this assay can now be routinely performed in these laboratories with confidence that the effects of a xenobiotic will be seen and a reproducible effect level ascertained.

All of the assays listed in Tier I of the testing panel (Table 1) have fulfilled the stringent requirements described for the PFC assay with regard to threshold values and dose–response curves (i.e., sensitivity) as well as reproducibility. For several assays the ability to detect effects at low levels in treated animals was dependent upon obtaining optimal or close to optimal responses in control groups. This can be typified by examining the ability of mice exposed to DES to mount a DHR (Table 6). In Laboratory C, where the DHR was not performed optimally (control S.I. = 1.9), the assay failed to detect significant suppression following chemical exposure except in the high-dose group. However, when optimal DHR control values were obtained (Laboratories A and B), a statistically significant effect was observed in DES-treated mice at several dose levels. In contrast to DHRs, the laboratories obtained fairly reproducible lymphoproliferation data. The dose–response curves for the depressed MLR and Con A lymphoproliferative responses in DES-treated mice, as well as resistance to *Listeria* infection (a reflection of DES-induced suppression of CMI), showed similar dose–

TABLE 6

INTERLABORATORY VALIDATION OF SELECTED IMMUNOLOGICAL ASSAYS USING DIETHYLSTILBESTROL

Parameter	Laboratory	Diethylstilbestrol (mg/kg) 0	0.1	1.0	4.0
Delayed hypersensitivity response index	A	4.72 ± 0.68	4.01 ± 1.38 (−15)[a]	2.06 ± 0.85 (−56)*	1.74 ± 0.51 (−63)*
	B	2.79 ± 0.17	2.79 ± 0.22 (0)	2.15 ± 0.20 (−23)*	1.88 ± 0.15 (−33)*
	C	1.88 ± 0.14	1.80 ± 0.14 (−5)	1.83 ± 0.16 (−3)	1.44 ± 0.11 (−24)*
Lymphoproliferative response cpm × 10^3					
Con A	A	156 ± 12	148 ± 6 (−6)	114 ± 10 (−27)*	92 ± 10 (−41)*
	B	145 ± 0	128 ± 9 (−9)	117 ± 9 (−19)	90 ± 8 (−48)*
	C	108 ± 4	98 ± 5 (−10)	75 ± 4 (−30)*	84 ± 6 (−23)*
LPS	A	68 ± 4	65 ± 7 (−4)	61 ± 3 (−10)	46 ± 4 (−33)*
	B	45 ± 3	ND	41 ± 2 (−8)	30 ± 5 (−33)*
	C	39 ± 3	36 ± 2 (−7)	30 ± 4 (−23)	28 ± 4 (−37)*
MLR	A	21 ± 1	20 ± 2 (+3)	7 ± 1 (−64)*	7 ± 1 (−66)*
	B	29 ± 2	19 ± 3 (−35)	9 ± 2 (−69)*	11 ± 3 (−63)*
	C	21 ± 1	21 ± 2 (0)	13 ± 2 (−62)*	13 ± 3 (−62)*
Listeria Mortality No. dead/No. tested	A	4/15 (27)	9/14 (64)*	11/15 (73)*	14/15 (93)*
	B	21/61 (34)	8/15 (53)	27/27 (100)*	34/39 (87)*
	C	4/20 (20)	19/20 (95)*	18/20 (90)*	15/20 (75)*

[a] Numbers in parentheses represent percentage change from control.
* $p < 0.05$ as compared to control group.

response effects in all three laboratories (Table 6).

Compared to immune function tests, the outcome of the interaction of an infectious agent or transplantable tumor cells with the host is more complex in that it is dependent upon such factors as antigenicity and virulence of the agents as well as host factors (e.g., strain, age, physiological status). Unlike the immune function assays where efforts were concentrated on refining existing assays, the host-resistance assays required extensive developmental work. Thus, less data are available comparing interlaboratory variations for the latter assays. Nevertheless, a number of infectivity models were examined and those listed in Table 1 demonstrated good intra- and interlaboratory reproducibility. A representative experiment is shown in Table 6 in which DES-exposed mice were monitored for their ability to resist challenge (LD20) with *L. monocytogenes*. Despite the many variables operative in host-resistance assays, the *Listeria* data consistently showed good correlation between the laboratories.

VI. CORRELATION BETWEEN ALTERED HOST RESISTANCE AND IMMUNE FUNCTION

Whether depression of the immune functions evaluated in these studies correlated qualitatively or quantitatively with altered susceptibility to challenge with an infectious agent or transplantable tumor was a major factor in ultimately judging the relevance of these immune function tests. It was likewise important to determine the significance of these measures if immunotoxicology data were to be used in risk assessment. As with most toxicological "indicators," it is assumed that certain functional changes in the immune system are predictive of significant

TABLE 7

CORRELATION BETWEEN HOST SUSCEPTIBILITY AND DEPRESSED IMMUNE FUNCTION

Challenge model	NK cytotoxicity	Proliferation		Antibody		
		MLR	PHA	LPS	PFC	DHR
PYB6 sarcoma	0.45[a,b]	0.46[b]	0.20	0.02	0.22	0.61[b]
B16F10 melanoma	0.54[b]	0.02	0.15	0.16	0.15	ND
Listeria	0.01	0.47[c]	0.37[b]	0.08	0.01	0.19
Influenza	0.11	0.78[b]	0.03	0.70[b]	0.83[c]	ND[d]
Plasmodium	0.24	0.59	0.67[b]	0.64[b]	0.78[c]	ND

[a] Correlation coefficient as determined by Spearman's rank correlation test (ρ values).
[b] Significant correlation at $p < 0.05$.
[c] Significant correlation at $p < 0.01$.
[d] ND, not done.

health effects. However, the degree of modulation is important since, as with other organ systems, a reserve capacity exists between a functional change and an increased risk to the host. Thus, depression of a particular function (e.g., lymphoproliferation or lymphocyte counts) beyond a critical point must occur if it is to result in altered host resistance. On the other hand, it is known that resistance to infectious agents involves a pleiotropic response such that loss of several immune functions, even to a slight degree, may lead to increased host susceptibility.

The relationship between immune dysfunction and altered susceptibility to challenge with infectious agents or transplantable tumors was analyzed for interdependence using Spearman's nonparametric rank correlation test (Lentner, 1982) with available data from exposed animals. Table 7 summarizes the results of this analysis for each immune function and challenge parameter where sufficient data were available for evaluation. Loss of the ability to generate PFCs in response to SRBCs and depressed lymphoproliferative responses to LPS correlated significantly with increased mortality following challenge with mouse-adapted influenza virus or with increased susceptibility to *Plasmodium*. Similarly, susceptibility to challenge with PYB6 sarcoma cells or B16F10 cells was significantly increased when NK cell function was reduced. There was no correlation observed between loss of NK cell activity and challenge with the infectious agents selected. Suppression of the MLR correlated with increased susceptibility to challenge with *L. monocytogenes, Plasmodium,* and PYB6 tumor cells, but not B16F10 melanoma cells. Finally, susceptibility to challenge with *L. monocytogenes* or *Plasmodium* increased significantly with the diminished responses to the T-cell mitogen, PHA. Other studies have also shown a correlation between depression of certain immune functions and increased susceptibility to challenge with infectious agents and transplantable tumor cells employing a similar configuration of functional assays (Dean *et al.*, 1987). In the latter studies, depression of NK cell function, generation of cytotoxic T cells, and the proliferative response to alloantigens in the MLR correlated with increased susceptibility to challenge with PYB6 transplantable tumor cells and the bacterial agent *L. monocytogenes.*

VII. QUALITY CONTROL AND DATA ANALYSIS

In this systematic evaluation of methods, it was necessary to establish a quality control program to monitor for assay variation or

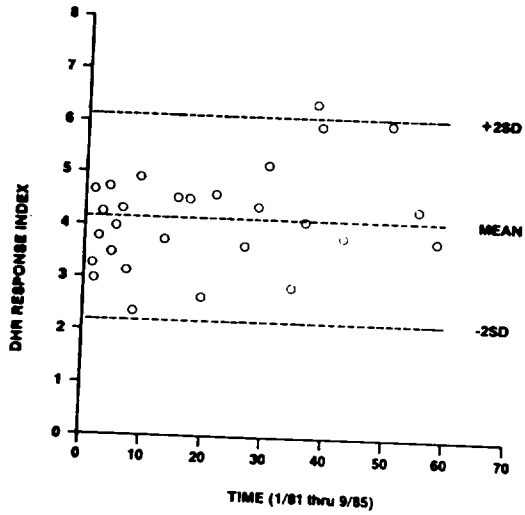

FIG. 2. Quality control plot showing delayed hypersensitivity response index to KLH in different groups of previously sensitized B6C3F1 mice tested over a 4-year period. Values are expressed as mean control response and 95% confidence interval (±2 SD).

drift. To accomplish this, the responses of normal animals were plotted over time as the mean ± 2 standard deviations (SD) to obtain a 95% confidence interval. By developing this approach, frequently used in clinical hematology and chemistry laboratories, it was possible to determine whether each assay performed within specifications during the course of the immunological assessment. On this basis, an objective decision as to the reproducibility (and validity) of an assay was obtained. An example of this type of exercise is shown in Fig. 2 where the DHR index is plotted versus time. Historical control data of this type have been assembled for all the assays as part of the quality control program. When performing chemical evaluation, those experiments in which the mean control values are more than 2 standard deviations from the historical mean are repeated.

For statistical analysis, immunological data were initially tested for homogeneity using Bartlett's χ^2 Test. For data that were determined to be homogeneous, one-way analysis of variance (ANOVA) was conducted. If the ANOVA was significant at $p < 0.05$ or less, Dunnett's multiple range t test was used for multiple treatment–control comparison. If the data were not homogeneous, nonparametric analysis of variance, the Kruskal–Wallis test, or the Wilcoxon rank sum test was used to compare treatment groups with control groups. The level of statistical significance was determined at $p < 0.05$ and $p < 0.01$. Values are routinely presented as mean ± standard error. For host-resistance data, χ^2 analysis or log linear models were used to determine chemical treatment effects on mortality. For comparisons of group survival times, the product limit estimator was used in conjunction with the Mantel–Cox test. Details of these analyses can be found elsewhere (Gad and Weil, 1982; Kaplan and Meier, 1958; Cox, 1972).

VIII. CONCLUSION

In summary, a comprehensive testing panel composed of two tiers has been developed and validated which is currently being used to characterize immune alterations that occur following *in vivo* chemical exposure in mice. In addition to sensitivity and reproducibility, the immunological assays utilized have been examined for correlations with alterations in host resistance, i.e., to challenge with infectious agents or tumor cells. For the most part, the assays detected clear dose–response effects with a no-observable-effect level being obtained for each prototype chemical examined. The correlation observed between functional measurements and challenge models, albeit preliminary, begins to provide insight into the biological significance of each of the various measures of immune function studied. Hopefully, this type of analysis will provide a better understanding of the relevant immune effector mechanism(s) involved in host resistance. Additional qualitative and quantitative information in this area should improve the accuracy with which the effects of chemicals or drugs on the immune system can be predicted.

These data also demonstrate the utility of the immune system for studying chemical

toxicity at the cellular level and as a sensitive indicator for chemical toxicity. Routine parameters measured in toxicology studies, such as blood or tissue cellularity, are often considerably less sensitive indicators of toxicity than are immune function measurements. Cells of the immune system possess a number of characteristics that make them appropriate models for examining chemical-induced cellular toxicity, including their capacity to undergo rapid proliferation or division *in vitro* following activation with antigen or nonspecific stimuli, their expression of gene products that can be utilized as markers of maturation, and their potential to undergo terminal differentiation resulting in production of measurable soluble mediators (e.g., monokines, lymphokines, or antibody) or providing effector functions (e.g., tumor cell killing).

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